Canaigre Investigations

VII. Fermentation of Extract Liquors, and Identity of the Bacteria and Products of their Growth*†

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INTRODUCTION

Consumption of vegetable tanning materials in this country amounts to about 140,000 tons of 100 per cent tannin per year. Almost two-thirds of this amount is imported. About 90 per cent of the domestic supply comes from American chestnut wood, almost all of which consists of trees killed by the chestnut blight.

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Because of this highly unsatisfactory condition, the U. S. Department of Agriculture has undertaken the development of domestic sources of tannin [7].

One of the most promising plants is canaigre (*Rumex hymenosepalus*, Torr.), a member of the dock family. This plant, native to the southwest United States, can be cultivated and harvested with machinery used for other crops. Satisfactory yields of roots can be produced from planted crowns or roots in 1-year, and a 2-year crop from seed is equal to two 1-year crops from crowns or roots.

Previous attempts to utilize canaigre as a source of tannin and some of the reasons for their failure are discussed by Rogers and Russell [12]. Table I

TABLE I

Composition of Representative Wild and Cultivated Canaigre Roots
(Moisture-Free Basis)

Source	Tannin	Total Sugars*	Starch	Purity†
	Per Cent			
Wild red (Arizona) roots	36.3	9.4	30.1	69.1
" " " "	42.2	9.2	22.2	71.8
Wild yellow (New Mexico) roots	23.6	19.5	21.4	47.7
" " " " "	22.7	20.5	20.8	45.1
Cultivated red roots	38.2	13.0	19.8	64.0
" " "	36.8	11.7	23.0	65.5
Cultivated yellow roots	21.6	9.9	35.1	45.7
" " "	22.3	11.2	33.5	46.9

*After acid hydrolysis.
†Tannin divided by soluble solids x 100.

gives the composition of some representative mature canaigre roots. These data illustrate the type of roots usually produced, but there is considerably more variation in actual samples than is shown. The red roots usually contain more tannin and have higher purity values than the yellow. From the data available, it appears that cultivated canaigre contains more than 35 per cent tannin. The economical production of canaigre as a tannin crop, however, will probably depend on utilization of the starch and sugars.

The presence of large proportions of starch makes the efficient extraction of tannin from canaigre roots by the usual leaching procedures impractical. A laboratory procedure has been developed, however, which gives satisfactory extraction yields [3]. Pilot-plant investigations with this procedure are being carried out.

An objection to canaigre as a source of tannin is that its low purity makes it unsuitable for production of heavy leather, for which most vegetable tannins are used (purity is tannin/soluble solids x 100). The low purity is due mainly to large amounts of sugar. A fermentation which destroys the sugars but leaves the tannin unaffected has been developed [4]. By this procedure, tanning extracts of high tannin content and purity have been made which

produce excellent heavy leather. The bacteria used in these fermentations were isolated from canaigre roots or soils in which the roots had grown. Yeasts and bacteria such as *Clostridia*, *Lactobacilli* and *Bacilli* failed to grow in canaigre liquors even though attempts were made to acclimate them.

The investigation reported in this paper was carried out (1) to identify the bacterial isolates, (2) to determine the fermentation products with a view to possible economic usefulness, and (3) to study conditions affecting the fermentation.

Identification of the Bacteria

The only microorganisms isolated which are able to grow in and ferment canaigre liquors are yeasts* of the genera *Endomycopsis* and *Torulaspora*, which, unfortunately, destroy tannin, and small gram-negative rod-shaped bacteria. These bacteria (12 isolates) were carried through the usual diagnostic procedures. Four known cultures were included for comparison. On the basis of these tests and the descriptions given in Bergey's Manual [1] three cultures would be *Aerobacter cloacae* except that they fermented lactose slowly. This places them in the *Paracolobactrum aerogenoides* group. The remaining nine cultures were *Aerobacter aerogenes* with only slight variations from the type species.

When first isolated, all twelve cultures actively fermented canaigre liquors. After laboratory culture in canaigre liquor for several months, this activity was lost to a marked degree by some isolates. Others have maintained their full activity for more than 4 years. The *Paracolobactrum aerogenoides* cultures produced slimy growth in canaigre liquors and lost their ability to actively ferment the liquors after laboratory cultivation.

Four known cultures obtained from the Northern Regional Research Laboratory (NRRL Nos. B199, B562, B492 and B499) grew in canaigre liquors, but their growth was slower and sugar decomposition was less complete than with our active isolates.

Experimental Results

Procedure

Canaigre liquors containing approximately 5 per cent solids, of which about one-half was tannin, were prepared by extracting finely ground roots by the method of Cordon, Beebe and Rogers [3]. Two types of roots were used: the Arizona or "red" type and the Texas-New Mexico or "yellow" type. Differences in their fermentability have been pointed out [4]. The liquors were heated for 15 to 30 minutes at 80° to 100°C., killing all organisms capable of growing. All spores were not destroyed, but no growth was found in liquors treated in this manner.

*Identified by L. J. Wickerham.

Inocula were prepared by growing the bacteria in small portions of the liquor to be fermented. The fermentations were carried out in wide-mouthed jars equipped with sampling tubes and fritted glass spargers. Aeration rates were measured with a wet test meter. The air was humidified by bubbling through water and sterilized by passage through activated carbon.

Chemical Methods

The work of Harden and Walpole [8] showed that *Aerobacter* species produce mainly 2,3-butanediol, and smaller quantities of acetoin, ethanol and organic acids. Ward, Pettijohn, Lockwood, and Coghill [15] have shown that *A. aerogenes* species produce a meso-dextro mixture of the diol, the former predominating, in contrast to the levo isomer produced by species of *Bacillus polymyxa*. We have presumed that the same form of 2,3-butanediol, and the same secondary products are produced in the fermentation of canaigre liquors by these strains of *Aerobacter*.

The progress of the fermentations was followed by determining reducing sugars (after hydrolysis, since about two-thirds of the sugar is sucrose), 2,3-butanediol, acetoin and ethanol. Refractive index measurements with an Abbé refractometer have been useful for following the progress of the fermentations and estimating the point of complete disappearance of sugar.

Sugars were determined by the method of Somogyi [14] after removing the tannins and proteins with basic lead acetate and hydrolyzing the deleaded solution with hydrochloric acid.

Butanediol was determined on an aliquot of a distillate made by distilling a portion of the liquor with tetralin* (tetrahydronaphthalene). A periodic acid oxidation method essentially that of Johnson [9] and an apparatus adapted from that of Edwards [5] were used in the determination. Corrections were made for acetoin.

Acetoin* was determined spectrophotometrically on an aliquot of the same tetralin distillate. The amount of acetoin was measured from the blue color produced by the reaction of the acetoin and the Folin-Wu phosphomolybdic reagent with a photometer and a wave length of 680 mu. No correction for possible interfering substances was found necessary.

Ethanol* was determined from the refractive index of a distillate obtained with a glass-packed fractionating column. A differential refractometer described by Brice and Speiser [2] was used for making the measurements. An empirical correction was applied for diacetyl produced from the acetoin present by its reaction with alkaline copper reagent, which was added to the distillation flask. Known mixtures of ethanol and acetoin were distilled, and

*Unpublished procedure developed at the Northern Regional Research Laboratory. These methods were checked using pure compounds and found to give accurate results. Details of the procedures may be obtained from this laboratory or from the Northern Regional Research Laboratory, Peoria, Illinois.

curves drawn from the data obtained. Knowing the acetoin content of the fermented liquors, we determined from the curves the apparent alcohol values due to acetoin and subtracted them from the alcohol values.

Results are reported here as grams per 100 ml of liquor and as per cent of theoretical based on sugar consumed. The theoretical value, 0.5 of the sugar consumed, was obtained by making certain assumptions which are outlined by Ledingham, Adams and Stanier [10].

Culture Evaluation

To determine whether these isolates behave similarly to known cultures of *A. aerogenes*, several of each were grown in a medium devised by Perlman [11]. The aeration rate was arbitrarily set at one-fifth volume of air/volume of liquor/minute with an incubation temperature of 30°C. Table II shows typical results. Culture WH consumed all the sugar present in the first 24 hours, after which time the reducing power of the fermented medium increased. These sugar values have not been corrected for acetoin, which also has reducing power. However, even if this correction is made, only part of the increase in reducing power is accounted for. Obviously some other reducing substance is produced. It seems reasonable to suspect that the oxidation might have proceeded one step further to the formation of diacetyl, which also reduces the copper reagent used in the sugar determinations. The butanediol and alcohol content of the medium was highest at the time of complete disappearance of sugar. As the fermentation continued, part of the butanediol was converted to acetoin, and the alcohol content decreased markedly. At the end of 72 hours' incubation, there was considerably more acetoin than butanediol present. This conversion of the butanediol to acetoin has been observed by other investigators [6, 13]. As the fermentation continued, the products were further metabolized, presumably to gaseous products. At the time the sugars had just been completely consumed, the nongaseous products amounted to approximately 90 per cent of theoretical; after a further incubation period of 48 hours they amounted to only 58 per cent.

The loss of sugar with culture B199 was considerably slower than with the other two cultures listed. The highest butanediol value was at the 24-hour incubation period but it was only about two-thirds as high as with cultures WH and B25, and the sugar was only 69 per cent consumed. It is possible that a higher butanediol content was reached between the 24- and 48-hour samplings. As with the other cultures, the proportion of ethanol was high (almost half) in the early stages of the fermentation.

Table III shows the formation of nongaseous products in the fermentation of red canaigre liquor by several strains of these bacteria. Fifty-eight to 67 per

TABLE II

Products of the Fermentation* of a Nutrient Medium† by Three Strains of *A. Aerogenes*

Culture No.	Fermentation Time, hours	Sugar Consumed		Products, g./100 ml			Products, Per Cent of Theoretical Based on Sugar Consumed		
		g./ 100 ml	Per Cent of original	Butanediol	Acetoin	Ethanol	Butanediol	Acetoin	Ethanol
B199	24	1.17	69.2	0.35	none	0.17	59.8	none	29.1
	48	1.57	92.9	0.32	0.11	0.15	40.8	14.0	19.1
	72	1.63	96.4	0.14	0.05	0.09	17.2	6.1	11.0
WH	24	1.69	100.0	0.52	none	0.24	61.5	none	28.4
	48	1.46	100.0	0.26	0.25	0.04	30.8	29.6	4.7
	72	1.38	100.0	0.19	0.27	0.03	22.5	31.9	3.6
B25	16	1.94	99.0	0.54	none	0.22	55.7	none	22.7
	40	1.94	99.0	0.40	0.09	0.18	41.2	0.9	18.6

* Aerated at the rate of 0.2 vol. of air/vol. medium/min.

†The medium contained peptone, 10 g; KH_2PO_4, 0.5 g; Na_2HPO_4, 2.0 g; $MgCl_2$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; and urea 3.0 g per liter with different amounts of added sucrose. (Medium of Perlman 11).

TABLE III

Products of the Fermentation* of Red Canaigre Liquor by Various Strains of *A. Aerogenes*†

Culture No	Fermentation Time, hours	Sugar Consumed		Products, g./100 ml			Products, Per Cent of Theoretical Based on Sugar Consumed		
		g./100 ml.	Per Cent of original	Butanediol	Acetoin	Ethanol	Butanediol	Acetoin	Ethanol
WH	16	0.81	44.5	0.27	0.04	0.03	66.7	10.0	7.4
	40	1.55	85.2	0.50	0.18	0.06	64.5	23.2	7.7
B25	24	1.14	69.9	0.36	0.02	0.04	63.2	3.5	7.0
	48	1.35	82.8	0.38	0.16	0.06	56.3	23.7	8.9
BNCT	15	1.22	74.9	0.41	0.02	0.07	67.2	3.3	11.5
	39	1.40	85.9	0.45	0.23	0.02	64.3	32.9	2.9
SR2	24	1.44	87.8	0.43	0.17	0.05	59.7	23.6	6.9
	48	1.33	87.8	0.28	0.21	0.06	38.9	29.2	8.3
B499	24	1.44	87.8	0.42	0.13	0.01	58.3	18.1	1.4
	48	1.31	87.8	0.26	0.17	0.06	36.1	23.6	8.3

*Aerated at the rate of 0.2 vol. air/vol liquor/min.
†Strains B199, HC, BSU, B21 SR3 and RD made only slight growth or failed to grow in this test.

cent of theoretical butanediol was produced in 15 to 24 hours. As in the nutrient medium, much of the butanediol was converted to acetoin in the later stages of the fermentation, but considerably less ethanol was formed than in the nutrient medium. With cultures WH and B25, the total yield of nongaseous products was much higher than in the nutrient medium at a comparable fermentation time. The yield in the canaigre liquor was approximately 90 to 100 per cent of the theoretical amount at 40–48 hours, as compared with about 60 to 65 per cent in the nutrient medium.

In no case was the sugar as determined by reduction completely consumed. This is invariably the case with canaigre liquors. There is always a residue of non-fermentable reducing matter, the nature of which has not been determined. It varies considerably with different lots of roots.

The results obtained with "yellow" canaigre liquor (Table IV) show the same general trends as those obtained with the "red" liquor. Except for culture BNCT, however, the fermentation rate was slower, and except for culture B492, the theoretical yield of products was somewhat lower.

From these results, it is apparent that the various strains of *A. aerogenes*, while giving the same general type of fermentation, vary considerably as to the rate at which they ferment canaigre liquors and the quantities of the materials they produce. Culture WH was selected for more detailed study because it has remained active and has not produced variants in more than 4 years of laboratory cultivation. Other cultures may prove to be equally good.

Factors Affecting the Fermentation

Liquors from both red and yellow canaigre roots were used in the following studies. The red roots appear to be superior to the yellow roots in tannin content, purity, clarity of liquors, and fermentability. Assuming equal yields on a tannin basis, the red roots would be preferable for cultivation.

Temperature

Most investigators who have studied *Aerobacter* fermentations have employed incubation temperatures between 28° and 35° C. The optimum is usually considered to be about 30° C. In canaigre liquors, these cultures are rather heat sensitive. Ten minutes at 65° C. almost completely inactivates them. In occasional failure of temperature-control equipment in pilot plant tests, the temperature has reached 40° C. In these cases the fermentation was markedly retarded. Laboratory tests (Table V) showed that at 25° C. the fermentation was considerably slower than at 30 or 35° C. However, the conversion to gases was more complete; no acetoin was formed, and only half as

TABLE IV

Products of the Fermentation* of Yellow Canaigre Liquor by Various Strains of *A. Aerogenes*

Culture No.	Fermentation Time, hours	Sugar Consumed		Products, g./100 ml			Products, Per Cent of Theoretical Based on Sugar Consumed		
		g/100 ml	Per Cent of original	Butanediol	Acetoin	Ethanol	Butanediol	Acetoin	Ethanol
B25	19	1.11	56.9	0.35	0.03	0.02	63.1	5.4	3.6
	43	1.52	78.0	0.36	0.16	none	47.4	21.1	none
BNCT	19	1.73	88.7	0.58	0.04	0.08	67.1	4.6	9.2
	43	1.66	88.7	0.37	0.18	0.02	42.8	20.8	2.3
BSU	20	1.25	64.1	0.26	0.01	0.07	41.6	1.6	11.2
	44	1.71	87.8	0.37	0.12	0.07	43.3	14.0	8.2
B492	20	0.19	9.7	0.10	none	none	105.2	none	none
	44	1.70	87.2	0.59	0.02	0.09	69.4	2.4	10.6
B199	20	0.43	22.1	0.10	none	0.01	46.5	none	4.7
	44	1.19	61.0	0.39	0.02	0.06	65.5	3.4	10.1

* Aerated at the rate of 0.2 vol. air/vol. liquor/min.

TABLE V

Effect of Temperature on the Fermentation*, † of Red Canaigre Liquor by *A. Aerogenes* No. WH.

Fermentation Temp. °C.	Sugar Consumed		Products g./100 ml.			Products, Per Cent of Theoretical Based on Sugar Consumed		
	g./100 ml.	Per Cent of original	Butanediol	Acetoin	Ethanol	Butanediol	Acetoin	Ethanol
25	1.22	76.7	0.32	none	0.06	52.5	none	9.8
30	1.49	93.7	0.42	0.11	0.13	56.4	14.8	17.4
35	1.48	93.1	0.40	0.13	0.14	54.1	17.6	18.9

* Aerated at 0.2 vol. air/vol. liquor/min.
† Incubation time, 18 hours.

much alcohol was produced. There was little difference between the 30° and 35° C. incubation temperatures.

Aeration

Table VI shows the effects of the aeration rate on the fermentation of yellow canaigre liquor by *A. aerogenes* No. WH. In stationary unaerated flasks, only 39 per cent of the sugar was consumed in 40 hours. A relatively high proportion of ethanol but little acetoin was produced. The butanediol produced was 63 per cent of theoretical, and about 85 per cent of theoretical nongaseous products were present. In the shaken flasks 59 per cent of the sugar was consumed in 40 hours. Less ethanol was produced but more acetoin. With aeration, the sugar was consumed much faster. Little ethanol but somewhat more acetoin was formed. The total yield of products was low and decreased with the volume of air and incubation time.

Table VII shows the results with red canaigre liquor under similar conditions, but with much more efficient shaking. Under anaerobic conditions, only about 9 per cent of the sugar was consumed in the 21-hour incubation period. Conversion to nongaseous products was efficient; 91 per cent of theoretical butanediol and 7 per cent of ethanol were formed. No acetoin was detected. All the shaken and aerated cultures fermented, at least to completion and probably beyond. There was little difference in the three aerated cultures. Extensive conversion of butanediol to acetoin had taken place, and almost an equal amount of ethanol was present. In each case more than 90 per cent of theoretical nongaseous products were formed.

To obtain more detailed information concerning the effect of aeration on this fermentation, air flow rates of one-fifth volume/volume of liquor/minute and one volume/volume of liquor/minute were run simultaneously and sampled at frequent intervals. The results are plotted in Figures 1 and 2. The usefulness of refractive index readings for following the course of the fermentations is illustrated. These values decreased until the sugar reached a minimum, re-

TABLE VI

Effect of Rate of Aeration on the Fermentation of Yellow Canaigre Liquor by *A. Aerogenes* No. WH

Aeration	Fermentation Time hours	Sugar Consumed		Products, g./100 ml.			Products, Per Cent of Theoretical Based on Sugar Consumed		
		g./100 ml.	Per Cent of original	Butanediol	Acetoin	Ethanol	Butanediol	Acetoin	Ethanol
None	40	0.73	39.2	0.23	0.01	0.07	63.0	2.7	19.2
Shaken*	40	1.09	58.6	0.35	0.04	0.09	64.2	7.3	16.5
0.2 vol. air/ vol. liquor/min.	18	0.97	52.1	0.24	0.05	0.02	49.5	10.3	4.1
"	22	1.10	59.1	0.25	0.06	0.02	45.5	10.9	3.6
"	40	1.64	88.1	0.35	0.06	none	42.7	7.3	none
1 vol. air/vol. liquor/min.	18	1.13	60.1	0.22	0.04	none	38.9	7.1	none
"	22	1 36	73.1	0.27	0.03	none	39.7	4.4	none
"	40	1.64	88.1	0.25	0.06	0.01	30.5	7.3	1.2

* Improvised from a bottle shaker. Rotated at 90 revolutions per minute.

TABLE VII

Effect of Rate of Aeration on the Fermentation of Red Canaigre by *A. Aerogenes* No. WH. Fermentation Time, 21 Hours

Aeration	Sugar Consumed		Products g./100 ml.			Products, Per Cent of Theoretical Based on Sugar Consumed		
	g./100 ml.	Per Cent of original	Butanediol	Acetoin	Ethanol	Butanediol	Acetoin	Ethanol
None	0.14	8.8	0.064	none	0.005	91.4	none	7.1
Shaken	1.32	83.0	0.30	0.17	0.06	45.5	26.0	9.1
0.2 vol. air/ vol. liquor/min.	1.35	84.9	0.37	0.16	0.13	54.8	23.7	19.3
0.5 vol. air/ vol. liquor/min.	1.36	85.5	0.36	0.15	0.11	52.9	22.1	16.2
1 vol. air/vol. liquor/min.	1.34	84.3	0.33	0.16	0.14	49.3	23.9	20.9

mained constant for a short period, then decreased again as the fermentation continued. With the slower aeration rate (Figure 1), the concentration of

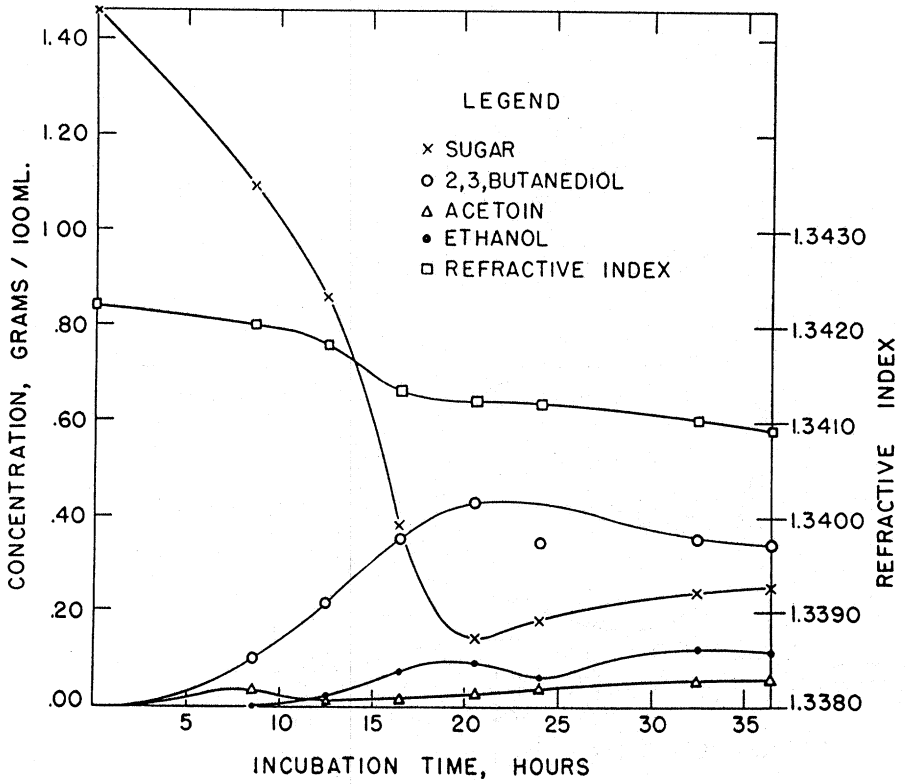

FIGURE 1.—Fermentation of red canaigre liquor with *A. aerogenes*, No. WH, aerated with one-fifth vol. air/vol. liquor/min.

sugar decreased to a minimum at 20.5 hours, then (the reducing action) increased gradually. The butanediol concentration was at a maximum when the sugar concentration was at a minimum. A gradual decrease followed. Only a small amount of ethanol and less acetoin were produced.

Increasing the air flow 5-fold (Figure 2) resulted in a more rapid loss of sugar and then a greater increase in reducing action, and less production and more rapid loss of butanediol. It had little effect on ethanol and acetoin.

Added Nutrients

The kinds and amounts of supplemental nutrients which may be added to canaigre liquors are limited. Proteinaceous materials are precipitated by the tannins, the pH may not be altered except slightly because of the adverse effect on the tannins, and any excessive amounts of salts contaminate the

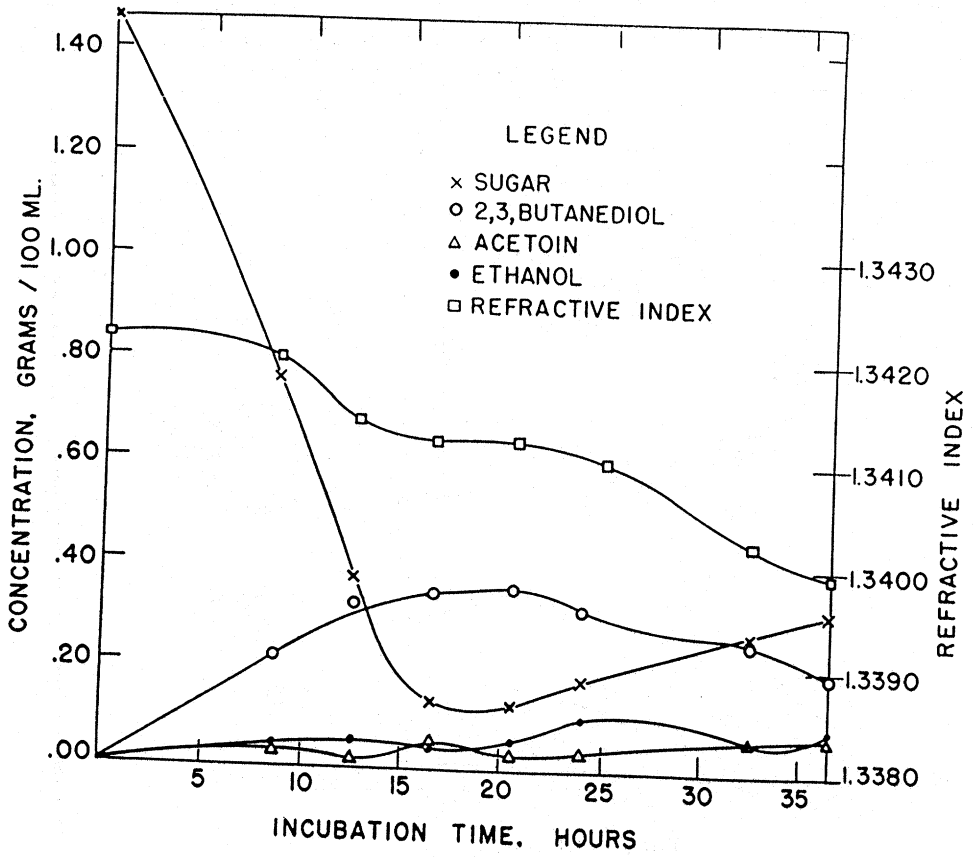

FIGURE 2.—Fermentation of red canaigre liquor with *A. aerogenes*, No. WH, aerated with one vol. air/vol. liquor/min.

liquors, since, after the fermentation, they are concentrated about 20-fold. However, small amounts of salts could probably be tolerated. Tables VIII and IX show the results obtained when certain nitrogen and phosphorus containing salts were added to red canaigre liquor. Ammonium nitrate appears to decrease the consumption of sugar somewhat, suppress production of alcohol and increase formation of butanediol. Urea increased the loss of sugar and the amount of acetoin formed at the expense of butanediol. Probably in this case the fermentation had gone beyond the time of complete sugar consumption. With dipotassium phosphate, slightly more butanediol and alcohol but less acetoin were formed than in the control. A combination of ammonium nitrate and dipotassium phosphate gave about the same results as ammonium nitrate alone, but produced slightly more butanediol and less acetoin.

TABLE VIII

Effect of Nutrient Salts on the Fermentation* of Red Canaigre Liquor by *A. Aerogenes* No. WH

Nutrients Added	Fermentation Time, hours	Sugar Consumed		Products, g./100 ml.			Products, Per Cent of Theoretical Based on Sugar Consumed		
		g./100 ml.	Per Cent of original	Butanediol	Acetoin	Ethanol	Butanediol	Acetoin	Ethanol
None, control	20	1.29	87.2	0.32	0.08	0.04	49.6	12.4	6.2
Ammonium nitrate 0.1%	20	1.02	68.9	0.33	0.04	none	64.7	7.8	none
Urea—0.1%	16	1.26	84.9	0.29	0.18	0.01	46.0	28.6	1.6
Dipotassium phosphate 0.05%	18	1.32	89.2	0.37	0.06	0.06	56.1	9.1	9.1
Ammonium nitrate 0.1%+ dipotassium phosphate 0.05%	18	1.06	71.6	0.36	0.02	none	67.9	3.8	none

* Aerated at 0.2 vol. air/vol. of liquor/min.

TABLE IX

Effect of Nutrient Salts on the Fermentation* of Red Canaigre Liquor by *A. Aerogenes* No. WH. Fermentation Time, 16 Hours

Nutrients Added	Sugar Consumed		Products, g./100 ml.			Products, Per Cent of Theoretical Based on Sugar Consumed		
	g./100 ml.	Per Cent of original	Butanediol	Acetoin	Ethanol	Butanediol	Acetoin	Ethanol
None, control	1.40	88.1	0.37	0.11	0.14	52.9	15.7	20.0
Urea 0.1% + Dipotassium phosphate 0.05%	1.39	87.4	0.41	0.12	0.14	59.0	17.3	20.1
Sodium nitrate 0.1%	0.80	50.3	0.13	0.07	0.03	32.5	17.5	7.5
Diammonium phosphate 0.1%	1.36	85.5	0.37	none	0.14	54.4	none	20.6

* Aerated at 0.2 vol. air/vol. liquor/min.

Urea and dipotassium phosphate together resulted in the formation of more butanediol and ethanol and less acetoin than when urea alone was used (Table IX). The total yield of these products was almost 100 per cent of theoretical, whereas in the control it was 88 per cent and with urea alone only 76 per cent. The dipotassium phosphate appeared to suppress the decomposition of the nongaseous products of the fermentation.

Sodium nitrate markedly decreased the amount of sugar utilized. The conversion to gaseous products, however, was greatly increased. Diammonium phosphate gave almost the same degree of fermentation as the control, and produced identical amounts of butanediol and ethanol. However, it produced no acetoin, and consequently the yield of nongaseous products was low.

Discussion

The fermentation of canaigre liquors by *A. aerogenes* was similar to the fermentation of other materials reported in the literature. Several differences, however, were apparent. The time required to metabolize a given amount of sugar was considerably longer in canaigre liquors, and there was a tendency toward more complete conversion to gaseous products. Ward, Pettijohn and Coghill [16] have reported yields of 86 per cent of theoretical butanediol from hydrolyzed corn mash. The usual yields in canaigre liquors were somewhat less and depended to a certain extent on the conditions of aeration, the strain of the organism used and the type of liquor.

Sugar in the liquors usually amounted to 1.5 to 2.0 per cent. In the red liquors, the fermentation was complete in 12 to 20 hours, whereas in yellow liquors more than 40 hours were usually required.

The ratio of butanediol to acetoin in fermenting liquors varied with the fermentation time. Stahly and Werkman [13] have shown that with *Bacillus polymyxa* the oxidation-reduction potential determines whether acetoin will be formed. They found that reducing conditions existed until almost all the sugar had been used, even though oxygen was bubbled through the medium, and under these conditions little acetoin was produced. Freeman [6] found that in the fermentation of sucrose by *A. aerogenes* acetoin was not formed until the sugar concentration fell below 1 per cent. Our results appear to agree essentially with those cited. No appreciable acetoin accumulation took place until the sugars had been largely utilized.

The situation with respect to ethanol is not clear. In some experiments, considerable ethanol was formed under anaerobic conditions. In others, it was formed at the expense of the butanediol when air was supplied. Nutrient salts, temperature and the type of liquor appeared to affect its formation. As with the butanediol and acetoin, it was further metabolized after the sugar had been consumed.

Acids formed during the fermentation were not determined because time did not permit carrying out the involved procedure necessary. That some

acids were produced is shown by the fact that the pH of fermenting liquors usually decreased slightly from the normal 4.5 to 5.5 during the early stages of the fermentation. A decrease in acidity then took place; if the incubation was continued long after the sugar was metabolized, the pH sometimes reached 7.5.

Summary

Bacterial isolates capable of fermenting canaigre liquors were identified as *Aerobacter aerogenes* or closely related species.

2,3-Butanediol is the main nongaseous product of the fermentation of canaigre liquors by these bacteria. Ethanol and acetoin are also produced, and if the fermentation continues after most of the sugar is consumed, the butanediol may be largely converted to acetoin.

Aeration markedly increases the fermentation rate but decreases the yield of products.

Some nutrient salts influence the fermentation rate and also appear to affect the ratio of the various products formed.

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